Evidence for markers of hypoxia and apoptosis in explanted human carotid atherosclerotic plaques

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Objective: **Apoptosis and inflammation are important features of atherosclerotic plaques. We investigated whether a common signal molecule can trigger these two apparently separate pathways. Hypoxia inducible factor (HIF-1α) is known to participate in atherosclerosis and to stimulate apoptosis signal-regulating kinase 1 (ASK-1), one of the mitogen-activated protein kinases, which is activated by various extracellular stimuli and involved in a variety of cellular function.**

Methods: **We tested carotid artery specimens from 50 subjects who underwent angioplasty and five age-matched controls for either Western blot or histologic analysis. The hypoxic status was investigated by means of HIF-1α expression in carotid specimens.**

Results: **HIF-1α was significantly upregulated in carotid specimens with respect to controls ($P < .05$), ASK-1 was detected in plaques of any composition from lipidic to calcific, and this expression increased with the stage of the plaque and with the expression of inflammatory (p-ERK, RANK-L, OPG) and apoptotic molecules (caspase 9, p-p-38, and p-JNK).**

Conclusion: **Our data suggest that hypoxia is the key regulating factor that triggers inflammation as well as apoptosis in the human atherosclerotic plaque. (J Vasc Surg 2010;52:1015-21.)**

Clinical Relevance: **This study adds evidence that HIF-1α is important in the oxidative stress response in the developing atherosclerotic plaque as it modulates both inflammatory and mitochondrion-related apoptotic pathways. Future therapy targeting HIF-1α may limit atherosclerosis development.**

A crucial moment in atherogenesis is thought to occur when the remaining population of functional phagocytes becomes unable to engulf all apoptotic remnants of dying cells in the environment.[1,2] Once the lesion contains a large, necrotic, lipid pool that is covered by a fibrous cap, primarily consisting of smooth muscle cells and extracellular matrix, it is named an atheroma.

In advanced atherosclerosis, death of macrophages in the setting of defective phagocytic clearance of apoptotic cells contributes to the development of plaque necrosis. Plaque necrosis, in turn, is thought to promote plaque disruption and arterial thrombosis, which are the proximate causes of acute cardiovascular events.[3,4]

Apoptosis signal-regulating kinase-1 (ASK1), a mitogen activated protein kinase, has been initially identified as a pro-apoptotic kinase. However, recently, ASK1 has been reported to also be implicated in a variety of cellular functions, including cell proliferation, survival, differentiation, and inflammatory response.[5-7] Moreover, it has been previously reported that ASK1 is the critical signalling molecule for angiotensin II-induced cardiac hypertrophy and remodelling[8] vascular neointimal thickening induced by balloon injury[9] and hind limb ischemia induced angiogenesis.[10] Thus, ASK1 seems to be involved in a variety of cardiovascular injuries. However, there is no report concerning the role of ASK1 in human carotid plaques.

Various stimuli, such as TNFα, Fas, and especially oxidative stress, activate ASK1, which induces apoptosis or stress responses through both JNK and p38 pathways activating the mitochondrial caspase-dependent apoptosis.[11] Especially, the ASK1-Trx complex is thought to be a redox-sensor, which functions as a molecular switch of external and internal redox status for the kinase signaling module. Multiple death signals are integrated into ASK1. In turn, the signals are transduced to mitochondria and nucleus, through the ASK1-JNK/p38 pathway. The balance between cellular life and death may be determined by timing, duration, extent, and pattern of the ASK1-mediated JNK and p38 activations, to make appropriate responses to various types of extracellular and intracellular stress.[12] Apoptosis in atherosclerotic plaques can be activated by either oxidative stress that causes hypoxia or shear stress that causes a persistent inflammatory status.

It has been recently shown that the late stage of the atherosclerotic plaque calcification is affected by the balance between osteoprotegerin (OPG) and receptor activa-

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Competition of interest: none.

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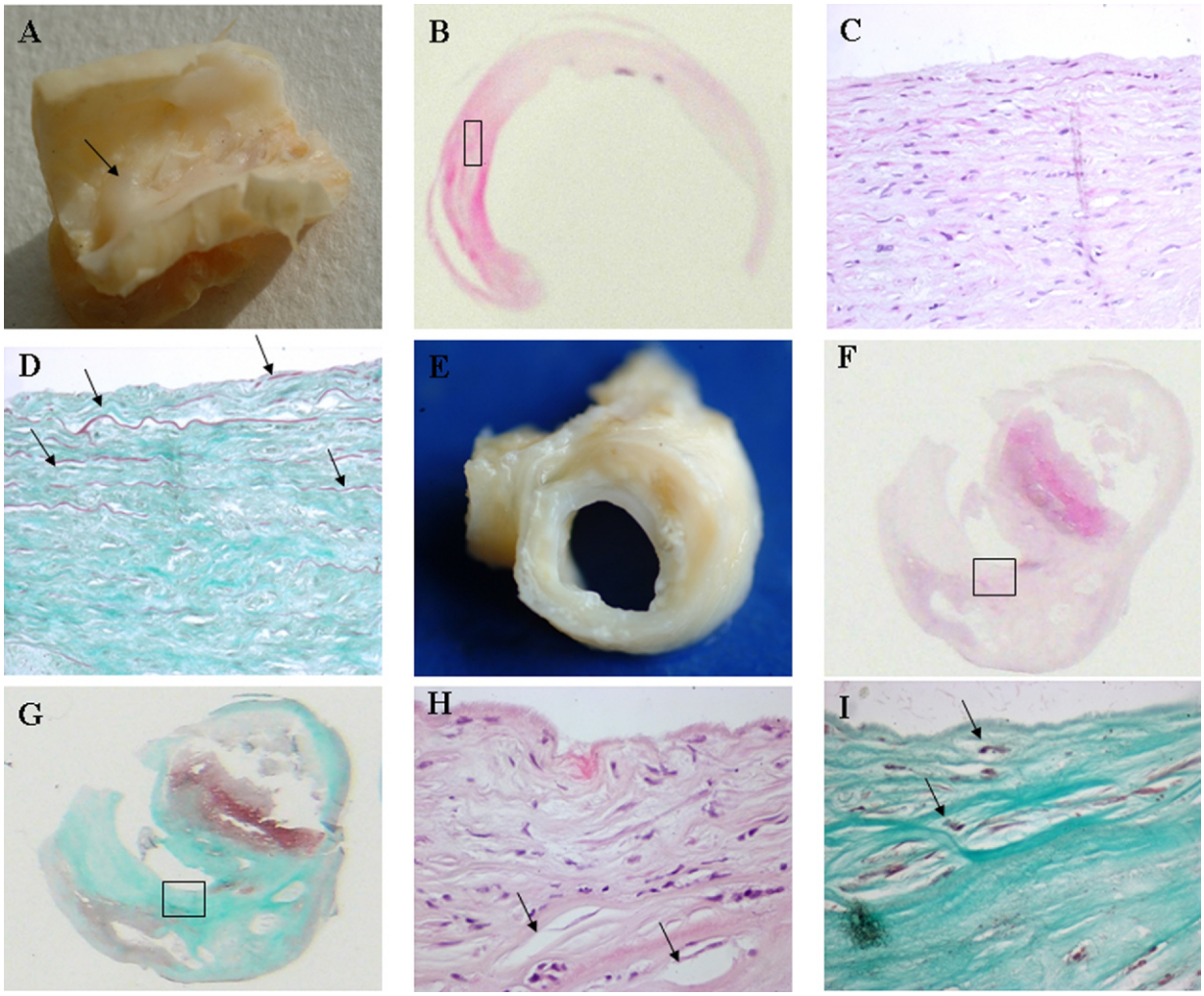

Fig 1. A, Macroscopic appearance of a normal carotid artery. Arrow head indicates an early fatty streak. **B,** H&E staining used for routine tissue preparation, nuclei stain *blue*, while connective tissue stains *pink*. The rectangle represents the area studied in **C** and **D**. Original magnification ×2. **C,** H&E staining, detail showing a normal arterial morphology. Original magnification ×20. **D,** Masson's trichrome staining, collagen fibers stain *green*, while muscle fibers stain *red*. *Arrowhead* indicates muscular fibers, *green color* indicates collagen tissue. Original magnification ×20. **E,** Macroscopic appearance of an atheromatous common carotid artery. **F,** H&E staining. The *rectangle* represents the area studied in H. Original magnification ×2. **G,** Masson's trichrome staining. The *rectangle* represents the area studied in I. Original magnification ×2. **H,** H&E staining, detail showing an altered morphological architecture with inflammatory infiltrate. *Arrow head* indicates adipose tissue deposition. Original magnification ×20. **I,** Masson's trichrome staining, *arrowheads* indicates calcification, and green color indicates fibrose tissue. Original magnification ×20.

tor of NF-kappa B ligand (RANK-L), which are well-known actors in bone metabolism but have been recently indicated as determinants of plaque calcification as well.[13] Moreover, RANK-L can be stimulated by inflammatory cytokines such as IL-1β and TNF-α.[14]

In light of these observations, the aim of our study was to investigate in human carotid plaques specimens, the expression of some markers of hypoxia and inflammation to better understand if a common pathway can be identified for these two different cascades.

MATERIALS AND METHODS

Patients and surgical procedures. All procedures were evaluated and approved by the ethics committee of the University of Messina, and the study procedures complied with the Declaration of Helsinki. Fifty consecutive patients were enrolled and signed an informed consent form before surgery. Patients have been selected based on plaque composition as determined by duplex ultrasound, to obtain an equal number of subjects for each plaque type

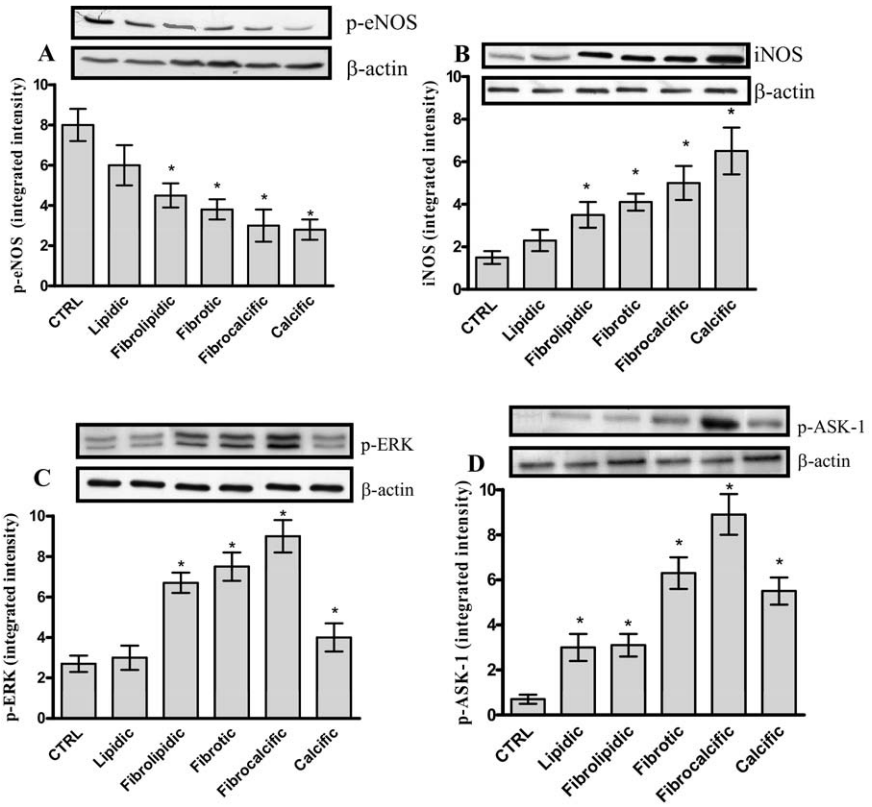

Fig 2. A, Representative Western blot of p-eNOS in carotid arteries. Bars represents mean ± SD. Controls n = 5. All the other groups n = 10. *$P < .05$ vs controls. **B,** Representative Western blot of p-eNOS in carotid arteries. Bars represents mean ± SD. Controls n = 5. All the other groups n = 10. *$P < .05$ vs controls. **C,** Representative Western blot of p-ERK in carotid arteries. Bars represents mean ± SD. Controls n = 5. All the other groups n = 10. *$P < .05$ vs controls. **D,** Representative Western blot of p-ASK-1 in carotid arteries. Bars represents mean ± SD. Controls n = 5. All the other groups n=10. *$P < .05$ vs controls.

(n = 10 for each type). Principal risk factors were hypertension (90%), diabetes (36%), dyslipidemia (48%), and smoking habit (34%). Mean age was 74 years.

Five control carotid arteries were removed at autopsy from age-matched car crash victims with the consent of a legal representative. Before the operation, all patients underwent duplex ultrasound to determine the extent of the atherosclerotic plaque as well as the composition of the plaque that was further confirmed by a pathologist. Indication for surgery in asymptomatic patients (40%) was a carotid stenosis of about 80%, according to the results of Asymptomatic Carotid Atherosclerosis Study (ACAS) trial;[15] in symptomatic patients (60%) indication for surgery was according to the results of North American Symptomatic Carotid Endarterectomy Trial (NASCET)[16] and European Carotid Surgery Trial (ECST)[17] studies.

The patient was placed in a supine position and a 7-10 cm incision was made along the anterior border of the sternocleidomastoid. After clamping, the internal carotid artery (ICA) was transected obliquely proximal to the bulb and a full thickness circumferential mobilization of the ICA was performed close to the distal clamp. Near the end point, the plaque was pinched and removed from the vessel for a total extension of 2-2.5 cm. The plaque on the external carotid artery (ECA) was removed by traction and partial eversion 1-1.5 cm from the origin of the ECA itself. The plaque on the common carotid artery (CCA) was extracted by eversion and transection of the plaque flush with the everted edge 1-1.5 cm from the bifurcation along the CCA.

Histology. Analysis was performed by a blinded investigator. For microscopic histologic evaluation, carotid specimens were removed and immediately fixed in 10% neutral buffered formalin. The specimens were placed in decalcifying solution (8% of chloridric acid 37% and 10% of formic acid 89% in PBS) at 37°C, dehydrated in graded ethanol, and then embedded in paraffin. Three 5-μm-thick paraffin-embedded sections were cut, dyed with a hematoxylin-eosin or Masson's Trichrome stain and studied using light microscopy.

Immunohistochemistry. Paraffin-processed sections were deparaffinized and rehydrated in graded ethanol solutions. Slides were then rinsed in distilled water and treated with 3% hydrogen peroxide in methanol (10 minutes at room temperature) to remove endogenous peroxidase ac-

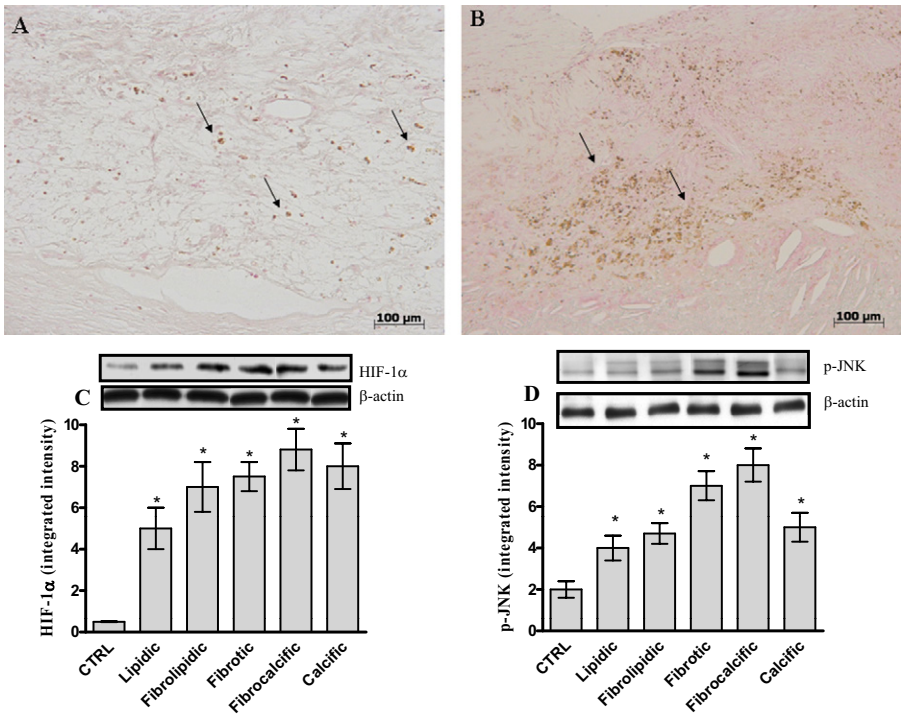

Fig 3. A, HIF-1α immunostaining in fibrocalcific carotid artery. Arrow heads shows nuclear reactivity. Original magnification ×40. $P < .01$ vs controls. **B,** p-JNK immunostaining in fibrocalcific carotid artery. Arrow heads shows diffuse cytoplasmic reactivity. Original magnification ×40. $P < .01$ vs controls. **C,** Representative Western blot of HIF-1α in carotid arteries. Bars represent mean ± SD. Controls n = 5. All the other groups n = 10. *$P < .05$ vs controls. **D,** Representative Western blot of p-JNK in carotid arteries. Bars represents mean ± SD. Controls n = 5. All the other groups n = 10. *$P < .05$ vs controls.

tivity. Sections were placed in Triton-X 100 for 20 minutes before blocking with goat antiserum (30 minutes at room temperature). Primary antibodies HIF-1α (Chemicon, Temecula, Calif) and anti-pJNK (Cell Signaling, Beverly, Mass) were then added and sections were incubated overnight at 4°C. After rinsing in PBS, standard Vectastain (ABC) avidin-biotin peroxidase complex (Vector Laboratories, Burlingame, Calif) was applied, and the slides were incubated at room temperature for a further 30 minutes. Color was developed using diaminobenzidine and sections were dehydrated, cleared, and mounted. Specificity of the immunoreaction was confirmed by a lack of immunostaining in control sections, in which the primary antibody was replaced with either PBS or pre-immune serum, and the secondary antibody was replaced by an irrelevant antibody or PBS (data not shown).

Western blot analysis. Samples from carotid plaques were homogenized in lysis buffer (1% Triton; 20 mM Tris/HCl, pH 8.0; 137 mM NaCl; 10% glycerol; 5 mM EDTA; 1 mM phenylmethylsulfonyl fluoride; 1% aprotinin; 15 μg/mL leupeptin). Protein samples (30 μg) were denatured in reducing buffer (62 mmol Tris/HCl, pH 6.8, 10% glycerol; 2% SDS; 5% β-mercaptoethanol; 0.003% bromophenol blue) and separated by electrophoresis on a SDS (12%) polyacrylamide gel. The separated proteins were transferred on to a nitrocellulose membrane using the transfer buffer (39 mM glycine, 48 mM Tris/HCl pH 8.3, 20% methanol) at 200 mA for 1 hour. The membranes were stained with Ponceau S (0.005% in 1% acetic acid) to confirm equal amounts of protein and blocked with 5% non-fat dry milk in TBS-0.1% Tween for 1 hour at room temperature, washed three times for 10 minutes each in TBS-0.1% Tween, and incubated with a primary antibody for HIF-1α, phospho-ASK-1, active caspase-9, RANK-L and OPG (Chemicon); phospho-p-38, phospho-JNK and phospho-e-NOS (Cell Signaling); and i-NOS (Abcam, Cambridge, UK) in TBS-0.1% Tween overnight at 4°C. After being washed three times for 10 min each in TBS-0.1% Tween, the membranes were incubated with a specific peroxidase-conjugated secondary antibody (Pierce, Rockford, Ill) for 1 h at room temperature. After washing, the membranes were analyzed by the enhanced chemiluminescence system according to the manufacturer's protocol (Amersham, Little Chalfont, UK). The protein signal was quantified by scanning densitometry using a bio-image analysis system (Bio-Profil Celbio, Milan, Italy). The results were expressed as relative integrated intensity compared with controls and subtracting respective backgrounds. More in detail the background is represented by β-actin expression that is used to normalize the results of densitom-

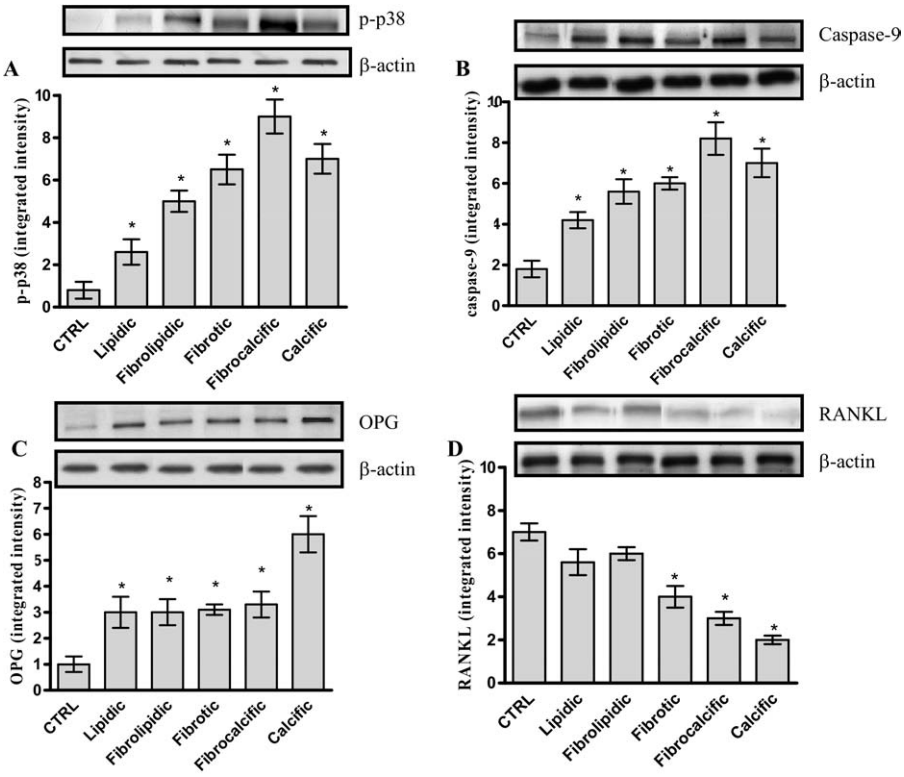

Fig 4. A, Representative Western blot of p-p38 in carotid arteries. Bars represent mean ± SD. Controls n = 5. All the other groups n = 10. **P* < .05 vs controls. **B,** Representative Western blot of active caspase-9 in carotid arteries. Bars represent mean ± SD. Controls n = 5. All the other groups n = 10. **P* < .05 vs controls. **C,** Representative Western blot of OPG in carotid arteries. Bars represents mean ± SD. Controls n = 5. All the other groups n = 10. **P* < .05 vs controls. **D,** Representative Western blot of RANKL in carotid arteries. Bars represent mean ± SD. Controls n = 5. All the other groups n = 10. **P* < .05 vs controls.

etry analysis. Equal loading of protein was assessed on stripped blots by immunodetection of β-actin with a rabbit monoclonal antibody (Cell Signaling) and peroxidase-conjugated goat anti-rabbit immunoglobulin G (Pierce). Antibodies were purified by protein A and peptide affinity chromatography. All experiments were repeated at least twice, and a representative example is shown.

Statistical analysis. All data are expressed as means ± standard deviation (SD). The significance of difference was assessed by a two-way repeated measures analysis of variance (ANOVA) followed by post hoc analyses. In all cases, a probability error of less than .05 was selected as the criterion for statistical significance. Graphs were performed using GraphPad Prism (version 4.0 for Windows).

RESULTS

Histologic classification of plaques. Histology confirmed plaque composition as determined by ultrasound in all cases. Furthermore, it was possible to subdivide plaques according to the main representative features into five phenotypes: lipidic (foam cells with lymphocytes), fibrolipidic (a relatively acellular, loose arrangement of collagen fibers), fibrotic (acellular area of dense collagen fibers), fibrocalcific (dense collagen fibers with calcific deposits), and calcific (heavily calcified tissue with or without spots of necrosis).

Carotid segments from control subjects showed only an early fatty streak that can be considered more than normal considering the age of the subjects (Table). Fig 1, *A* through *D*, represents a carotid artery from a control subject with an initial fatty streak and a conserved intima and media layers where collagen and muscular fibers are well visible. Fig 1, *E* through *I* , is a representative fibrocalcific plaque with initial ulceration in the core and loss of normal architecture.

Hypoxia induces inflammatory response in plaques. All the plaque phenotypes showed a hypoxic state as indicated by the progressive decrease in p-eNOS (Fig 2, *B*; control = 8 ± 0.8 integrated intensity, calcific = 2.8 ± 0.5 integrated intensity; $P < .05$) and the increase in iNOS (Fig 2, *C*; control = 1.5 ± 0.3 integrated intensity, calcific = 6.5 ± 1.1 integrated intensity; $P < .05$) expression in plaques, with respect to normal carotids. Because of the enhanced oxidative stress, a significant staining of nuclear HIF-1α has been found in plaques (Fig 3, *A*, $P < .01$ vs controls). This has also been confirmed by Western blot

(Fig 3, *C*) and likely triggered p-ERK as detected by Western blot (Fig 2, *D*; $P < .05$ vs controls).

Hypoxia induces apoptosis in plaques via ASK-1, JNK, p-38, and caspase-9. HIF-1α staining in atherosclerotic plaques was associated with p-JNK increase at the same site that was more prominent in fibrocalcific plaques (Fig 3, *B*; $P < .01$ vs controls), as well as an increase in p-ASK-1 (Fig 2, *E*; $P < .05$ vs controls), p-JNK (Fig 3, *D*; $P < .05$ vs controls), p-p38 (Fig 4, *A*; $P < .05$ vs controls), and active caspase-9 ($P < .05$ vs controls) as detected by Western blot (Fig 4, *B*).

Hypoxia triggers calcification markers expression. The end stage of atherosclerotic plaques is calcification, thus, we also investigated whether this process could be activated in earlier stages by the Western blot expression of OPG and RANKL. Interestingly, OPG was upregulated in plaques roughly in the same extent ($P < .05$ vs controls) with a peak of expression in calficic specimens (Fig 4, *C*). In the same way, a progressive decrease in RANKL expression has been observed in all specimens with respect to controls ($P < .05$ for fibrotic, fibrocalcific, and calcific plaques; Fig 4, *D*).

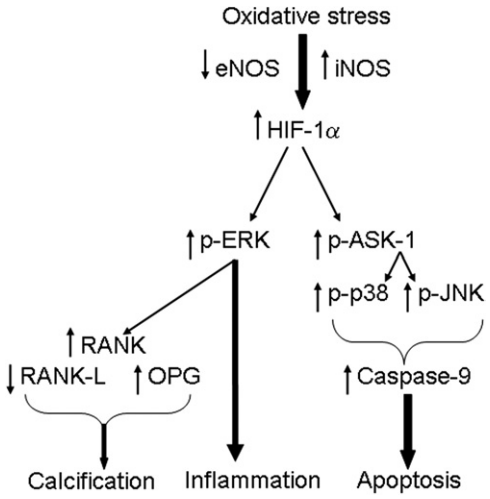

Fig 5. Proposed cascade of events triggered by oxidative stress in atherosclerotic plaque.

DISCUSSION

The onset of plaque formation is characterised by circulating levels of oxidized low-density lipoproteins, which are capable of penetrating the endothelial border while inflicting oxidative damage on the endothelial cells in the process. Subsequently, the reactionary expression of inflammatory markers on the endothelial cell surface attracts circulating monocytes, which differentiate into macrophages and start ingesting the available lipid and other oxidized particles. Uninhibited intracellular lipid accumulation compromises the function of macrophages and converts them into foam cells.[1] At a certain point, excess damage to macrophages and smooth muscle cells induces apoptosis, causing remnants of lipid-laden dead cells to form small extracellular lipid droplets.

The main findings of our study are the early expression of apoptotic molecules in the atherosclerotic plaque and the involvement of oxidative stress in triggering an inflammatory as well as an apoptotic response. These cascades might be linked through the activation of either HIF-1α and/or ASK-1 pathways (Fig 5); however, this hypothesis needs to be proved in further studies. It has already been shown that hypoxia and its related factor HIF-1α are present in athermatous plaques and this latter is correlated with plaque progression. HIF-1α also regulates NOS expression in plaques at different stages and eventually intraplaque angiogenesis[18] *via* the expression of VEGF genes by binding to the hypoxia response element in the VEGF promoter region. In the presence of oxygen, HIF-1α protein is rapidly degraded via ubiquitination and subsequent degradation by proteasome. Under hypoxia, HIF-1α is not hydroxylated and therefore cannot be degraded. Consequently, HIF-1α accumulates in the nucleus, forms an active complex with HIF-1β, and activates transcription of target genes. Furthermore, other authors correlated HIF-1α with the proinflammatory status of atherosclerotic plaques.[19] This supports our immunostaining results showing HIF-1α localization in either perivascular tissue or inflammatory macrophages. In addition, HIF-1α can stimulate the expression of ASK-1 leading to the activation of the mitochondrial apoptosis signalling pathway. We showed that HIF-1α expression correlates with NO products and iNOS expression, although it also triggers ASK-1 expression and caspase-9 activation through the JNK pathway. Caspase-9 is one of the initiator caspases responsible for mitocondrial apoptosis and has been already observed in macrophages surrounding the unstable plaque. The authors suggest this molecule to be a key determinant in plaque ulceration and rupture.[20] The authors also showed an increase in pro-apoptotic MAPKs as p38 and JNK. Indeed, we found the same increase in these molecules but we further showed an augmented expression of ASK-1, which is the leading activator of this pathway. The slight decrease in MAPKs activation observed in calcific plaques is consistent with a decrease of macrophages in these lesions due to calcification itself. All these are early events and are strictly connected in plaque development and further increased with plaque evolution as demonstrated. In addition, our data showing the activation of the mithocondrial apoptotic pathway, further confirm the findings of Sarai and coworkers in experimental animals treated with inhibitors of caspases belonging to both pathways.[11] In addition, it could be speculated that the rise in oxidative stress highlighted by the decreased eNOS expression and the consequent increase in iNOS might account for the activation of HIF-1α and for a proinflammatory status characterized by augmented ERK expression in the plaque. As we hypothesize, the common initiator is oxidative stress, which through HIF-1α in turn activates inflammation and apoptosis from the very early moments of plaque formation. Indeed, we also found that other molecules related to the

final stages of atheromasia, as RANK-L and its decoy receptor OPG, are expressed early. Indeed, p-ERK can upregulate RANK,[21] and this might in turn lead to a decrease in RANK-L and a consequent increase in OPG, as we have demonstrated. OPG expression suggests that, potentially, all plaques can go through the same cascade of events that lead to calcification and stabilization instead of ulceration and rupture. On the other hand, RANK-L and OPG serum levels have been proposed as biomarkers of vascular risk and prognosis correlating with the narrowing of the arterial wall[22] as well as with the severity of coronary artery disease.[23] It has been reported that these levels are elevated in myocardial infarction subjects,[24] suggesting that calcification is crucial for plaque destabilization and rupture.[25] However, whether the imbalance in OPG/RANK-L ratio is a consequence or a causal factor of plaque destabilization still deserves further investigation. Obviously, genetic background as well as medical therapy and lifestyle are responsible for a different story in plaque evolution. It becomes more evident that future therapies should be addressed to a modulation of HIF-1α, since it seems to be responsible for triggering both inflammatory and mitochondrion-related apoptotic pathways.

AUTHOR CONTRIBUTIONS

Conception and design: AB, FS, FSquadrito
Analysis and interpretation: AB, FP, DA
Data collection: GDC, FP, MC, NI
Writing the article: AB, FSquadrito
Critical revision of the article: AB, GDC, FSpinelli, FSquadrito
Final approval of the article: AB, GDC, FP, MC, NI, DA, FSpinelli, FSquadrito
Statistical analysis: AB, FP, MC, NI
Obtained funding: DA, FSquadrito
Overall responsibility: FSpinelli, FSquadrito

REFERENCES

1. Steinberg D, Lewis A. Conner Memorial Lecture. Oxidative modification of LDL and atherogenesis. Circulation 1997;95:1062-71.
2. Tabas I. Consequences and therapeutic implications of macrophage apoptosis in atherosclerosis: the importance of lesion stage and phagocytic efficiency. Arterioscler Thromb Vasc Biol 2005;25:2255-64.
3. Schrijvers DM, De Meyer GR, Herman AG, Martinet W. Phagocytosis in atherosclerosis: molecular mechanisms and implications for plaque progression and stability. Cardiovasc Res 2007;73:470-80.
4. Virmani R, Burke AP, Kolodgie FD, Farb A. Vulnerable plaque: the pathology of unstable coronary lesions. J Interv Cardiol 2002;15:439-46.
5. Ichijo H, Nishida E, Irie K, ten Dijke P, Saitoh M, Moriguchi T, et al. Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. Science 1997;275:90-4.
6. Hayakawa T, Matsuzawa A, Noguchi T, Takeda K, Ichijo H. The ASK1-MAP kinase pathways in immune and stress responses. Microbes Infect 2006;8:1098-107.
7. Ichijo H. From receptors to stress-activated MAP kinases. Oncogene 1999;18:6087-93.
8. Izumiya Y, Kim S, Izumi Y, Yoshida K, Yoshiyama M, Matsuzawa A, et al. Apoptosis signal-regulating kinase 1 plays a pivotal role in angiotensin II-induced cardiac hypertrophy and remodeling. Circ Res 2003;93:874-83.
9. Izumi Y, Kim S, Yoshiyama M, Izumiya Y, Yoshida K, Matsuzawa A, et al. Activation of apoptosis signal-regulating kinase 1 in injured artery and its critical role in neointimal hyperplasia. Circulation 2003; 108:2812-8.
10. Izumi Y, Kim-Mitsuyama S, Yoshiyama M, Omura T, Shiota M, Matsuzawa A, et al. Important role of apoptosis signal-regulating kinase 1 in ischemia-induced angiogenesis. Arterioscler Thromb Vasc Biol 2005; 25:1877-83.
11. Sarai M, Hartung D, Petrov A, Zhou J, Narula N, Hofstra L, et al. Broad and specific caspase inhibitor-induced acute repression of apoptosis in atherosclerotic lesions evaluated by radio labeled annexin A5 imaging. J Am Coll Cardiol 2007;50:2305-12.
12. Matsuzawa A, Ichijo H. Redox control of cell fate by MAP kinase: physiological roles of ASK1-MAP kinase pathway in stress signaling. Biochim Biophys Acta 2008;1780:1325-36.
13. Collin-Osdoby P. Regulation of vascular calcification by osteoclast regulatory factors RANKL and osteoprotegerin. Circ Res 2004;95: 1046-57.
14. Collin-Osdoby P, Rothe L, Anderson F, Nelson M, Maloney W, Osdoby P. Receptor activator of NF-κB and osteoprotegerin expression by human microvascular endothelial cells, regulation by inflammatory cytokines, and role in human osteoclastogenesis. J Biol Chem 2001; 276:20659-72.
15. Committee for the Asymptomatic Carotid Atherosclerosis Study Endarterectomy for asymptomatic carotid artery stenosis. JAMA 1995;273: 1421-8.
16. North American Symptomatic Carotid Endarterectomy Trial Collaborators. Beneficial effect of carotid endarterectomy in symptomatic patients with high-grade carotid stenosis. N Engl J Med 1991;325:445-53.
17. European Carotid Surgery Trialists. Collaborative Group. Medical Research Council European Carotid Surgery Trial: Randomized trial of endarterectomy for recently symptomatic carotid stenosis: final results of the MRC European Carotid Surgery Trial (ECST). Lancet 1998; 351:1379-87.
18. Luque A, Turu M, Juan-Babot O, Cardona P, Font A, Carvajal A, et al. Overexpression of hypoxia/inflammatory markers in atherosclerotic carotid plaques. Front Biosci 2008;13:6483-90.
19. Vink A, Schoneveld AH, Lamers D, Houben AJ, van der Groep P, van Diest PJ, et al. HIF-1 alpha expression is associated with an atheromatous inflammatory plaque phenotype and upregulated in activated macrophages. Atherosclerosis 2007;195:e69-75.
20. Slevin M, Elasbali AB, Miguel Turu M, Krupinski J, Badimon L, Gaffney J. Identification of differential protein expression associated with development of unstable human carotid plaques. Am J Pathol 2006;168:1004-21.
21. Lee ZH, Kwack K, Kim KK, Lee SH, Kim HH. Activation of c-Jun N-terminal kinase and activator protein 1 by receptor activator of nuclear factor κB. Mol Pharmacol 2000;58:1536-45.
22. Schoppet M, Sattler AM, Schaefer JR, Herzum M, Maisch B, Hofbauer LC. Increased osteoprotegerin serum levels in men with coronary artery disease. J Clin Endocrinol Metab 2003;88:1024-8.
23. Jono S, Ikari Y, Shioi A, Mori K, Miki T, Hara K, et al. Serum osteoprotegerin levels are associated with the presence and severity of coronary artery disease. Circulation 2002;106:1192-4.
24. Crisafulli A, Micari A, Altavilla D, Saporito F, Sardella A, Passaniti M, et al. Serum levels of osteoprotegerin and RANKL in patients with ST elevation acute myocardial infarction. Clin Sci (Lond) 2005;109:389-95.
25. Montecucco F, Steffens S, Mach F. The immune response is involved in atherosclerotic plaque calcification: could the RANKL/RANK/OPG system be a marker of plaque instability? Clin Dev Immunol 2007; 2007:75805.

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